Chapter 6

Yeast Two-Hybrid Screening to Test for Protein–Protein Interactions in the Auditory System

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Abstract

We describe a protocol to screen for protein–protein interactions using the Gal-4-based yeast two-hybrid system. In this protocol, we describe serial transformation of bait into an already constructed cDNA library in yeast AH109 cells. We find this method gives the most number of true interactions. Where a premade library in yeast cells is not available, the method outlined can be quickly adapted. AH109 cells can be first transformed with bait containing a vector followed by selection of yeast containing the bait. A second transformation of yeast cells is then accomplished with the cDNA library. The method is quick and can lead to the discovery of significant interactions.

Key words Protein–protein interactions, Yeast two-hybrid, Gal4-based, Cochlea cDNA library, pGBKT7, pGADT7, AH109, Serial transformation

1 Introduction

The yeast two-hybrid system was first developed to detect protein–protein interactions between two known proteins [1]. There are several different yeast two-hybrid systems available and several key papers and reviews have a detailed accounting [2–14]. Yeast two-hybrid systems include the GAL4-based system, the Lex-based system, and the split ubiquitin-based system [11, 14, 15]. In this chapter, we lay out our laboratory protocol on using the Gal4 yeast two-hybrid system. There are presently many exciting and newer uses of the yeast two-hybrid system, including mass screenings to globally define interacting proteins (interactome) [16–19].

A short theoretical description of the GAL4-based system follows, since this method is a recent one. Briefly, the GAL4-based assay described here is based on the fact that eukaryotic transcriptional activators consist of two parts, a DNA binding domain and a transcription activation domain, that need to be in close physical proximity with one another to initiate transcription. The DNA binding domain binds the promoter, whereupon the transcription

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activation domain "instructs" RNA polymerase to transcribe a downstream (reporter) gene. The transcripts encoding the protein of interest ("bait") are tagged to the binding domain and a mixture of different unknown proteins are tagged to the activation domain (cDNA). These are then co-transformed into yeast. Transcription ensues when the activation and binding domains are brought into close proximity, as a result of protein–protein interactions between the bait protein and an unknown second protein. By using a selectable marker downstream of the promoter, the yeast two-hybrid screen allows us to choose colonies, where an interaction has occurred between the bait protein and a second unknown protein. These colonies are then picked and grown, and the transcript encoding the protein that interacts with the bait protein is isolated and sequenced.

Described below is our laboratory protocol using the Matchmaker two-hybrid system from Clontech, a commercially available yeast two-hybrid system. This system uses the Gal4 activation and binding domains with several reporter genes (HIS3 and ,ADE2 α galactosidase and β galactosidase). The HIS3 and ADE2 gene products allow growth in medium lacking histidine and adenine and, also, permit us to screen with different degrees of stringency (i.e., strength of protein–protein interaction). The HIS3 marker allows for selection with medium stringency while the ADE2 marker allows for selection with high stringency. The galactosidase genes, of which the α form is secreted, allow for easy identification of interacting proteins by blue-white screening. A second feature in using the Matchmaker system is that the inserts and the bait are also epitope-tagged, allowing us to confirm protein–protein interaction in vitro.

In the protocol laid out herein, it is assumed that the user has access to a yeast cDNA library from the tissue of interest in AH109 cells. If not, a commercial library can be purchased and used to serially transform yeast. The yeast are then grown in differentially restrictive conditions (Synthetic dropout media (SD)—His—Ade, and Synthetic dropout media—His; *see* **Note 1**). The plates contain X-alpha-Gal that allows evaluation of galactosidase secretion.

Two methods can be used to introduce the two plasmids into a single yeast cell: chemical transformation and yeast mating. Chemical transformation involves physical incorporation of plasmid DNA into yeast cells, while yeast mating is dependent on haploid yeast cells mating and taking on a diploid configuration. The latter requires the presence of two complementary gene products (MAT and MAT alpha) in the two species of yeast. Yeast strain AH109contains MAT, whereas Y187 contains MAT alpha. While mating efficiency is high and allows a larger fraction of cells carrying the prey to be interrogated by the bait, the process still involves chemical transformation of yeast strains with the bait plasmid into one strain (usually Y187) and the prey plasmid into another strain (usually AH109), when premade libraries are not available.

2 Materials

2.1 Hardware

1. 150 mm plates.
2. Benchtop centrifuge and microcentrifuge.
3. 15 and 50 mL conicals.
4. 42 and 30 °C water bath.
5. Large platform rocker with speed and temperature controls for growing yeast.
6. Large platform rocker with speed and temperature controls for growing bacteria.
7. 30 °C incubator for yeast plates.
8. 37 °C incubator for yeast plates.
9. Spectrophotometer.
10. Vortexer.
11. Large platform rocker with speed and temperature controls.

2.2 Solutions

All reagents are prepared in Milli-Q water.

1. Yeast Extract Peptone Dextrose (YPD Medium): Prepare 1 L of medium by mixing 10.0 g of yeast extract (Difco, BD Biosciences, CA), 20.0 g of Peptone (Difco), 20.0 g of Glucose, in 950 mL of Milli-Q water. Adjust pH to 6.5 and bring volume to 1 L with water. Autoclave at 120 °C for 15 min.
2. Agar Plates: Mix YPD with 10 g of Bacto-agar (Difco), autoclave at 120 °C for 15 min.
3. 2× YPD Medium: Prepare 1 L of medium by mixing 20.0 g of yeast extract (Difco), 40.0 g of Peptone (Difco), 40.0 g Glucose, and 0.6 g of L-tryptophan in 950 mL of water. Adjust pH to 6.5 with 1 N HCl and bring volume to 1 L with water. Autoclave at 120 °C for 15 min.
4. SD (Synthetic Dropout) Medium: Mix 6.7 g of Yeast Nitrogen Base without amino acids, 20.0 g of Glucose, 100 mL of 10× Dropout Supplement in 950 mL of water. Adjust pH to 5.8 with 1 N HCl and bring volume to 1 L. Autoclave at 120 °C for 15 min.
5. 10× Complete Dropout Solution. Mix the following components in 1000 mL of Milli-Q water: 200 mg of L-adenine hemisulfate, 200 mg of L-arginine HCl, 200 mg of L-histidine HCl monohydrate, 300 mg of L-isoleucine, 1000 mg of L-leucine, 300 mg of L-lysine HCl, 200 mg of L-methionine, 500 mg of L-phenylalanine, 2000 mg of L-threonine, 200 mg of L-tryptophan, 300 mg of L-tyrosine, 200 mg of L-uracil, 1500 mg of L-valine.

6. 10× Dropout Solution (-Ade/-His/-Leu/-Trp). Mix the following components in 1000 mL of Milli-Q water: 200 mg of L-arginine HCl, 300 mg of L-isoleucine, 300 mg of L-lysine HCl, 200 mg of L-methionine, 500 mg of L-phenylalanine, 2000 mg of L-threonine, 300 mg of L-tyrosine, 200 mg of L-uracil, 1500 mg of L-valine.
7. 10× Dropout Solution (-His/-Leu/-Trp). Mix the following components in 1000 mL of Milli-Q water: 200 mg of L-adenine hemisulfate, 200 mg of L-arginine HCl, 300 mg of L-isoleucine, 300 mg of L-lysine HCl, 200 mg of L-methionine, 500 mg of L-phenylalanine, 2000 mg of L-threonine, 300 mg of L-tyrosine, 200 mg of L-uracil, 1500 mg of L-valine.
8. 10× Dropout Solution (-Leu). Mix the following components in 1000 mL of Milli-Q water: 200 mg of L-adenine hemisulfate, 200 mg of L-arginine HCl, 200 mg of L-histidine HCl monohydrate, 300 mg of L-isoleucine, 300 mg of L-lysine HCl, 200 mg of L-methionine, 500 mg of L-phenylalanine, 2000 mg of L-threonine, 200 mg of L-tryptophan, 300 mg of L-tyrosine, 200 mg of L-uracil, 1500 mg of L-valine.
9. 10× Dropout Solution (-Trp). Mix the following components in 1000 mL of Milli-Q water: 200 mg of L-adenine hemisulfate, 200 mg of L-arginine HCl, 200 mg of L-histidine HCl monohydrate, 300 mg of L-isoleucine, 1000 mg of L-leucine, 300 mg of L-lysine HCl, 200 mg of L-methionine, 500 mg of L-phenylalanine, 2000 mg of L-threonine, 300 mg of L-tyrosine, 200 mg of L-uracil1, 500 mg of L-valine.
10. 50% Polyethylene Glycol (PEG) Stock Solution: Dissolve 50 g of PEG 3350 in 100 mL of water. The solution may need to be heated to 55 °C to dissolve the polyethylene glycol.
11. 10× Li Acetate: Dissolve 6.6 g of LiAc in 85 mL of water and adjust pH to 7.5 with acetic acid. Autoclave at 120 °C for 15 min.
12. 10× TE buffer: Mix 12.1 g of Tris base, 3.72 g of ethylenediaminetetraacetic acid (EDTA, disodium salt) in 950 mL of water and adjust pH to 7.5 with 1 N HCl. Autoclave at 120 °C for 15 min.
13. PEG/Li Acetate solution: Mix 8 mL of 50 % PEG, 1 mL of 10× Tris-EDTA (TE) and 1 mL of 10× Li Acetate.
14. 3-Amino-1,2,4-triazole (3-AT)should be added to SD/-Trp at varying concentrations before plating cells with AH109 cells that are transformed with bait in pGBK T7 plasmid. Double transformants are then grown in SD/-His/-Leu/-Trp medium using the minimal concentration of 3-ATthat shows no growth of the bait plasmid transformed AH109 cells.
15. cDNA libraries. Chick cochlea cDNA libraries are available upon request. Please contact Dhasakumar.Navaratnam@Yale.Edu.

3 Methods

There are several different yeast two-hybrid systems available. These include the Gal4-based system, the Lex-based system, and the split ubiquitin-based system. In this chapter, we describe our protocol for the Gal4 yeast two-hybrid system.

3.1 Two-Hybrid Screening

3.1.1 Preparing the Bait Vector

1. Three methods are available for yeast two-hybrid screens: co-transformation, yeast mating, and serial transformation. Of these, we find serial transformation and mating to give the greatest number of transformants and potential interactions (*see* **Note 2**).
2. Subclone the fragment of cDNA, to be used as bait, into vector pGBK T7. Since the Gal4-based system depends on soluble proteins, it is important that the sequence of interest does not contain potentially membrane-spanning regions. Use PCR amplification to generate the cDNA fragments. Design primers complementary to the nucleic acid sequence in the fragment of cDNA with restriction fragments at their 5′ ends. Remember to add an extra 4–5 nucleotides at the 5′ end so that the restriction enzyme can "bite." Also, note that the 5′ end should have the protein of interest in frame with the *myc* epitope.

3.1.2 Testing for Leaky His3 Expression (See Note 3)

1. Streak AH109 (or Y187 if using yeast mating) on YPDA plates and grow until colonies appear (~2–4 days).
2. Pick isolated colony and grow in 5 mL of YPD medium overnight at 30 °C.
3. Transfer cells to 50 mL of YPD medium and continue growing at 30 °C.
4. Periodically assess Optical Density (O.D.) at 600 nm to test that the cells are growing.
5. Once O.D. 600 exceeds 0.15–0.3, centrifuge cells at 700 × *g* for 10 min at room temperature.
6. Resuspend cell pellet in 50 mL of 2× YPD.
7. Grow cells at 30 °C for 1–3 h with shaking in a 500 mL flask.
8. Once an O.D. 600 reading of 0.4 is attained, centrifuge cells at 700 × *g* for 10 min at room temperature and discard supernatant.
9. Resuspend cell pellet in 50 mL of water and centrifuge at 700 × *g* for 10 min at room temperature.
10. Resuspend cell pellet in 1.5 mL of 1.1× TE/LiAc solution centrifuge at 700 × *g* for 10 min at room temperature, and discard supernatant.
11. Resuspend cell pellet in 0.6 mL of 1.1× TE/LiAc solution.

12. In a 1.5 mL microfuge tube, mix 0.1 μg of bait plasmid in pGBKT7 (>0.5 μg/μL) and 10 μL of denatured sheared herring testes carrier DNA (10 mg/mL). To denature carrier DNA, add desired amount of DNA from Subheading 3.1.1, **step 3** to a 0.5 mL PCR tube and set thermal cycler to: 94 °C for 5 min, 4 °C for 5 min, 94 °C for 5 min, and hold at 4 °C.
13. Add 100 μL of AH109 cells from Subheading 3.1.2, **step 11**. Mix gently by vortexing, then add 0.6 mL of PEG/LiAc solution to each 1.5 mL microfuge tube.
14. Mix gently by vortexing, then incubate at 30 °C for 45 min. Mix cells every 15 min.
15. Add 70 μL of DMSO to each 1.5 mL microfuge tube and mix.
16. Place cells in 42 °C water bath for 15 min and mix cells every 5 min.
17. Centrifuge cells at 700 × *g* for 2 min at room temperature.
18. Resuspend in 3 mL of 2× YPD medium and incubate cells at 30 °C for 2 h with shaking.
19. Centrifuge cells at 700 × *g* for 2 min at room temperature.
20. Resuspend each pellet in 0.5 mL of 0.9 % NaCl.
21. Spread 50 μL cells each on two SD/-Trp/plates and two plates each of SD/-Trp/-His, containing 2.5, 5.0, 10, and 15 mM 3-amino-1,2,4-triazole and two plates of SD/-Trp/-His. If there is no growth on SD/-Trp/-His/plates without 3-amino-1,2,4-triazole, the reagent does not needed to be added to SD/-Leu/-Trp/-His in Subheading 3.1.3, **step 3**. If however, there is leaky His3 expression, add 3-amino-1,2,4-triazole to plates at a concentration where no growth is seen (*see* **Note 3**).

3.1.3 Materials Prepared the Day Before the Y2H Experiment

1. Prepare sufficient subcloned pGBKT7 vector containing cDNA for entire experiment (~10 μg). We use Qiaquick purified plasmid with no further purification.
2. Prepare media and plates the day before the planned experiment and set incubator temperature for 30 °C.
3. Prepare the following number of agar plates with specific dropout medium: 200 SD/-Ade/-Leu/-Trp/-His/X-Gal plates, 200 SD/-Leu/-Trp/-His/X-Gal plates, 4 SD/-Leu plates, 4 SD/-Trp plates, and 4 SD/-Leu/-Trp/plates. Add 3-amino-1,2,4-triazole to a final concentration determined in Subheading 3.1.2 to plates lacking His (-His/), where no growth is seen in previously conducted tests, as described in Subheading 3.1.2, **step 21**. *See* also **Notes 1** and **4**.

3.1.4 Conducting the Y2H Experiment

The following ten steps are performed on the morning of the Y2H experiment. We use chemical transformation that has given us the

most consistent results with good viability (and contrasts favorably with electroporation).

1. Thaw 2 mL of cDNA library (>5×10^7 colony forming units per mL) in AH109 cells at room temperature. The library contains the putative protein–protein partners or "prey."
2. Add entire contents of library into 100 mL of 2× YPD medium.
3. Grow cells at 30 °C for 1–3 h with shaking in a 500 mL flask.
4. Periodically assess O.D. 600 to determine if the cells are growing.
5. Once O.D. 600 exceeds 0.15–0.3, centrifuge cells at 700 × *g* for 10 min at room temperature.
6. Resuspend pellet in 100 mL of 2× YPD and grow cells at 30 °C for 1–3 h with shaking in a 500 mL flask.
7. Once an O.D. 600 reading of 0.4 is attained, centrifuge cells at 700 × *g* for 10 min at room temperature.
8. Resuspend cells in 80 mL of water, then centrifuge cells at 700 × *g* for 10 min at room temperature.
9. Resuspend cells in 3 mL of 1.1 × TE/LiAc solution, then centrifuge cells at 700 × *g* for 10 min at room temperature.
10. Resuspend pellet in 1.2 mL of 1.1 × TE/LiAc solution. Divide cells into two 0.6 mL aliquots.
11. Prepare two sterile 15 mL conical tubes by cooling and maintaining them on ice, then add in order: 3 μg of bait plasmid in pGBK T7 (>0.5 μg/μL) and 20 μL of denatured sheared herring testes carrier DNA (10 mg/mL). To denature carrier DNA, add desired amount of DNA to 0.5 mL PCR tube and set thermal cycler to: 94 °C for 5 min, 4 °C for 5 min, 94 °C for 5 min, then hold at 4 °C.
12. Add 600 μL of AH109 cells from Subheading 3.1.4, **step 10**.
13. Mix gently by vortexing, then add 2.5 mL of PEG/LiAc solution to each 15 mL conical tube.
14. Mix gently by vortexing then, incubate at 30 °C for 45 min. Mix cells every 15 min.
15. Add 160 μL of DMSO to each 15 mL conical tube and mix.
16. Place cells in 42 °C water bath for 20 min and mix cells every 10 min.
17. Centrifuge cells at 700 × *g* for 10 min at room temperature.
18. Resuspend each cell pellet in 3 mL of 2× YPD medium and incubate cells at 30 °C for 2 h with shaking.
19. Centrifuge cells at 700 × *g* for 10 min at room temperature, then resuspend cells in 30 mL of 0.9 % NaCl.
20. Spread 150 μL of cells from conical tube 1 onto each of the 100 150 mm plates containing SD/-Ade/-Leu/-Trp/-His/

X-Gal. Spread 150 μL of cells from conical tube 2 onto each of the 100 150 mm plates containing SD/-Leu/-Trp/-His/X-Gal. Spread 150 μL from each tube on two SD/-Leu/-Trp/plate, two SD/-Leu/plate, and two SD/-Trp/plates. Please also *see* **Note 4**.

21. Incubate for 4–8 days at 30 °C or until colonies appear. Colonies should appear by 2 days but no longer than 8 days (*see* **Notes 5–8**).

3.1.5 Yeast Mating Protocol

1. Pick a single colony of Y187 that has been chemically transformed with yeast bait vector (pGBK with bait insert). Inoculate 10 mL SD/-Trp with the single colony. Incubate overnight with shaking (200 RPM on a Brand?) at 30 °C.
2. Centrifuge cells at 700 × *g* for 10 min at room temperature.
3. Resuspend cells in 10 mL of fresh SD/-Trp and inoculate entire contents into 100 mL of fresh SD/-Trp medium. Incubate overnight with shaking (200 RPM) at 30 °C.
4. Centrifuge cells at 700 × *g* for 10 min at room temperature. Resuspend pellet in 100 mL of 2× YPD and place in a sterile 2 L flask.
5. Thaw out one 1.5 mL frozen vial of AH109, containing the prey library and inoculate entire vial into 2× YPD medium, containing Y187 GBK bait plasmid.
6. Incubate overnight at 30 °C with shaking at 50 RPM.
7. After 18 h of incubation remove 10 μL and examine under phase contrast to confirm mating. If mating is confirmed centrifuge cells at 700 × *g* for 10 min at room temperature. If mating is not confirmed incubate cells for another 4 h and centrifuge cells.
8. Resuspend cells in 31 mL TE. Centrifuge cells at 700 × *g* for 10 min at room temperature. Resuspend pellet in 10 mL of TE.
9. Spread 150 μL of cells onto each of the 100 150 mm plates containing SD/-Ade/-Leu/-Trp/-His/X-Gal. Spread 150 μL of cells onto each of the 100 150 mm plates containing SD/-Leu/-Trp/-His/X-Gal. Spread 150 μL from each tube on two SD/-Leu/-Trp/plate, two SD/-Leu/plate, and two SD/-Trp/plates. Please also *see* **Note 4**.

3.1.6 Picking Colonies and Controlling for Artifacts

1. Count the number of colonies in the SD/-Leu/-Trp/plate, SD/-Leu/plate, and SD/-Trp/plate to determine the number of double transformants (pGADT7 and pGBKT7), and transformants with pGBK T7 respectively. The number of colonies in SD/-Leu/plate will confirm the number of viable cells in the library (titer) (*see* **Note 4**).

2. Pick single colonies from each plate and re-streak on similar selective plates. That is, streak colonies from SD/-Ade/-Leu/-Trp/-His/X-Gal onto SD/-Ade/-Leu/-Trp/-His/X-Gal plates, and colonies from SD/-Leu/-Trp/-His/X-Gal onto SD/-Leu/-Trp/-His/X-Gal plates (*see* **Notes 5**–**8**).
3. Pick individual colonies from each of the new plates and grow in the corresponding restrictive media by inoculating 0.5 mL of medium with one colony each. That is colonies from SD/-Ade/-Leu/-Trp/-His/X-Gal to be grown in SD/-Ade/-Leu/-Trp/-His/medium, and colonies from SD/-Leu/-Trp/-His/X-Gal on SD/-Leu/-Trp/-His/medium.
4. Grow cells at 30 °C for 2 days with shaking at 200 RPM. .
5. Centrifuge cells at 700 × *g* for 10 min at room temperature.
6. Resuspend cells in 5 mL of sterile SD/-Trp/medium to isolate only yeast containing the pGADT7-cDNA.
7. Grow cells at 30 °C for 2 days with shaking. Remove 0.85 mL and add 0.15 mL of sterile glycerol. Freeze in nalgene cryovials at −80 °C. These stocks serve as a reservoir in the event of unforeseen errors.
8. Centrifuge remaining cells from Subheading 3.1.6, **step 7** at 700 × *g* and proceed to isolate DNA by yeast mini-prep.

3.2 Yeast DNA Isolation and Transformation of Bacteria

3.2.1 Isolation

We have attempted to isolate yeast DNA using a number of methods with variable success (*see* **Note 9**). We have now settled on using the Zymoprep I/II™ Yeast Plasmid Minipreparation Kit (Zymoresearch, Orange, CA), which gives us the most consistent results. The method is similar to the commonly used alkaline lysis method used with bacteria, but has an antecedent enzyme digestion step for breaking down the cell wall.

1. Centrifuge 1.5 mL of yeast liquid culture in a 2 mL Eppendorf tube.
2. Add 15 μL of Zymolyase™ for each 1 mL of Solution 1 to make a Solution 1-enzyme mixture.
3. Add 200 μL of Solution 1 to each pellet.
4. Add a further 3 μL of Zymolyase™ to each tube. Resuspend the pellet by mild vortexing and incubate at 37 °C for 60 min.
5. Add 200 μL Solution 2 to each tube. Mix well.
6. Add 400 μL Solution 3 to each tube. Mix well.
7. Centrifuge at 18,000 × *g* for 3 min.
8. Transfer the supernatant to the Zymo-Spin-I column.
9. Centrifuge the Zymo-Spin I column for 30 s at 14,000 × *g*.
10. Discard the flow-through in the collection tube. Make sure the liquid does not touch the bottom part of the column.

11. Add 550 μL of Wash Buffer (EtOH added) onto the column with the collection tube and centrifuge for 1 min at 18,000 × *g*.
12. Place column in fresh 1.5 mL microfuge tube and centrifuge at 18,000 × *g* for an additional 1 min.
13. Place column in fresh microtube, add 10 μL of water, and incubate for 5 min at room temperature. Elute plasmid off the column by centrifuging for 1 min at 18,000 × *g*.

3.2.2 Transforming Bacteria with Isolated Plasmid

The isolated DNA is used to transform bacteria (*see* **Note 10**). We find electroporation of the DNA (using the manufacturer's instructions) into *E. coli* DH10B (Invitrogen, Carlsbad, CA) gives the best results. Once *E. coli* are transformed, follow these steps.

1. Spread cells onto ampicillin plates using 5 mM glass beads.
2. Pick individual clones using a sterile toothpick to inoculate 3 mL of LB medium, containing 50 μg of Ampicillin/mL. Isolate DNA using a standard mini-prep kit (e.g., Qiagen plasmid mini-prep kit).
3. Determine the sequence of the inserted cDNA using the T7 primer. We use in-house or commercial sequencing services.

3.3 Confirming Interactions

Interactions demonstrated using the yeast two-hybrid system need to be confirmed by other techniques such as immunoprecipitation or FRET (*see* **Note 11**). While an in-depth description of these techniques is beyond the scope of this chapter, we will mention briefly the technique of coimmunoprecipitation. We routinely use in vitro synthesized S35 Methionine-labeled protein, as it is the most useful method. The WGA system from Clontech gives us the most consistent results. We are able to use the sequences in the pGBKT7 and pGADT7 vectors without further subcloning. The vectors can be linearized and used to run off RNA using the T7 promoter. Moreover, both vectors are designed to incorporate a *Myc* and Flag tag at the N-terminus of each inserted cDNA sequence. These tags allow for easy immunoprecipitation and visualization by autoradiography of the corresponding two proteins (confirming interactions).

Also, we follow immunoprecipitation experiments in vitro with experiments in vivo, using antibodies, where available, to the two interacting proteins. When antibodies to the native proteins are not available, we resort to epitope tagging the entire protein. The proteins are then expressed in HEK cells and separately immunoprecipitated. The converse protein is then detected in the immunoprecipitate by western blotting.

3.4 Where cDNA Libraries in Yeast Are Not Available

The method outlined here assumes that you have pGAD T7-cDNA libraries in yeast AH109 cells. This method of serial transformation can be used also to test for interactions using commercial non-yeast libraries. In this case, AH109 cells derived from a fresh single

colony should be made competent as indicated in Subheading 3.1.4, **steps 1–10**. The cells should be transformed with bait containing pGBKT7 and grown in SD/-Trp medium. A second round of transformation is then performed using the cDNA library in pGADT7 with the same protocol outlined above (*see* Subheading 3.1.4, **steps 1–21**). Following the second round of transformation, cells are plated on SD/-Ade/-Leu/-Trp/-His/ plates and SD/-Leu/-Trp/-His plates to isolate colonies with interacting proteins.

4 Notes

1. Readers may wonder why we limit ourselves to using relatively stringent conditions for detecting interacting proteins. In our experience, the real difficulty with yeast two-hybrid techniques is not one of sensitivity (number of interactions) but rather one of specificity (number of true interactions). We have therefore not had to use low stringency screens. In these screens yeast after transformation of bait and prey vectors are grown in SD/-Leu/-Trp/. After colonies are identified, they are re-plated on plates with increasing stringency (SD/-Ade/-His/-Leu/-Trp/ and SD/-His/-Leu/-Trp/).
2. We have tried using co-transformation, serial transformation, and yeast mating in trying to detect protein–protein interactions. Of these methods, our data indicate that serial transformation and mating give the most number of interacting proteins. The downside to these methods is that it is possible that the bait protein could be eliminated by recombination in the event that it is toxic to the cell.
3. Leaky His3 expression. At times it is possible that the bait in pGBK T7 can alone activate transcription of the His3 protein. The level of His3 expression is low and can be easily suppressed by the addition of 3-AT. We first test if the bait protein in pGBKT7 activates His3 expression in AH109 cells by transforming these cells with the bait pGBKT7 construct alone. These cells are then plated on SD/-Trp/-His with varying concentrations of 3-AT added to the medium. We then note the concentration of 3-AT at which there is no observed growth. We then add 3-AT to SD/-Trp/-Leu/-His plates used for medium stringency screening of the yeast two-hybrid screen. We do not add 3AT to the high stringency screen (SD/-Trp/-Leu/-His/-Ade).
4. Each of the constructs contains different reporter genes allowing one to select for cells containing that construct. For instance, the pGBKT7 vector contains the tryptophan reporter allowing cells containing this vector to be grown in medium lacking tryptophan. Likewise, the pGAD T7 vector that contains the

cDNA library has the Leucine reporter. Thus, cells containing this plasmid can be grown in medium lacking leucine. Double transformants (that is cells containing both pGBKT7 and pGAD T7) can be grown on media lacking both Leucine and Tryptophan. This must be contrasted with double transformants that show an interaction that will grow in medium lacking leucine, tryptophan, histidine, and adenine. The latter two are reporters that are dependent on an interaction between the GAL4 binding domain and GAL4 activation domain.

5. A feature that must be paid attention to is the shape and the color of yeast in plates incubated in restrictive media. AH109 cells after 2–4 days of growth appear as 2–5 mm sized plaques that resemble a small blob of yoghurt. There are no visible hyphae. Contaminant yeast from ambient air can sometimes grow on these plates, but their morphology makes them easy to identify.
6. The times of incubation in restrictive conditions (SD/-His/-Trp/-Leu/; SD/-Ade/-His -Trp/-Leu/) may vary and depends on the strength of the interacting proteins. For instance, weaker interactions may result in growth that is visible after 5–8 days. The criterion that we use is the size of the yeast colony—they must be 2 mm or larger. Often colonies are seen on day 2 that do not grow to be larger than 2 mm.
7. Colonies that express Ade are white or pale pink in color. Colonies that lack Ade turn brown to red in SD/-Ade/-His/-Trp/-Leu/medium.
8. In our screens, we use both alpha and beta galactosidase assays only at the point of secondary screening. The reason we do not use this assay in the initial screening is owing to cost. Although we do not use it as a method for detecting interactions the amounts of alpha and/or beta galactosidase can also be used as a test of the strength of protein–protein interactions. There are several plate and liquid assays (chromogenic and fluorescent) for both these enzymes.
9. We have attempted to isolate yeast DNA using a number of methods with variable success. We have now settled on using the Zymoprep I/II™ Yeast Plasmid Minipreparation Kit, which has given us the most consistent results. The method is similar to the commonly used alkaline lysis method used with bacteria, but has an antecedent enzyme digestion step for breaking down the cell wall.
10. Isolated DNA from yeast mini-preps should be used to transform bacteria. This will allow adequate amounts of DNA for subsequent experiments (DNA sequencing, making proteins for pull-down assays, subcloning into mammalian expression vectors, etc.). While the commonly used heat shock method can

be used, we find the amount of recovered DNA to be variable and at times too low to effectively transform bacteria. We have instead found electroporation (using the manufacturer's instructions) into *E. coli* DH10B (Gibco BRL Life Technologies, Inc.) to give the best results. Spread cells on ampicillin plates.

11. It is now widely recognized in the field that while yeast two-hybrid technology is a sensitive technique, it is susceptible to nonspecific artifacts. A number of reasons account for this including absent physiological significance, leaky reporters, etc., and are discussed in several of the reviews below. The bait protein too does influence this to some degree. Nevertheless, it is now de-rigueur to confirm interactions detected, using the yeast two-hybrid system, by immunoprecipitation. Additionally, in vivo and subcellular interactions can further be determined using fluorescence resonance energy transfer (FRET).

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